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[Title of the Invention] Method for Producing Target Substance by Fermentation

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[Title of Invention] Method for Producing Target Substance by Fermentation

5 [Claims]

[Claim 1] A  $\gamma$ -proteobacterium having an ability to produce a target substance and modified so that an ArcA protein does not normally function.

[Claim 2] The  $\gamma$ -proteobacterium according to claim 1, wherein the ArcA protein that normally functions is a protein defined in the following

10 (A) or (B):

(A) a protein having the amino acid sequence of SEQ ID NO: 18;

(B) a protein having the amino acid sequence of SEQ ID NO: 18 including substitution, deletion, insertion or addition of one or several amino acids and improving an ability to produce a target substance when the  
15 protein does not normally function in the  $\gamma$ -proteobacterium compared with the case where the protein normally functions.

[Claim 3] The  $\gamma$ -proteobacterium according to claim 1, wherein the ArcA protein that normally functions is a protein having 70% or more of homology to the amino acid sequence of SEQ ID NO: 18 and improving an ability to  
20 produce a target substance when the protein does not normally function in the  $\gamma$ -proteobacterium compared with the case where the protein normally functions.

[Claim 4] The  $\gamma$ -proteobacterium according to claim 1, wherein the ArcA protein that normally functions is a protein having the amino acid sequence  
25 of SEQ ID NO: 18 including substitution, deletion, insertion or addition of 2 to 20 amino acids and improving an ability to produce a target substance when the protein does not normally function in the  $\gamma$ -proteobacterium compared with the case where the protein normally functions.

[Claim 5] The  $\gamma$ -proteobacterium according to any one of claims 1 to 4,  
30 wherein the ArcA protein does not normally function by means of disruption

of an *arcA* gene on a chromosome.

[Claim 6] The  $\gamma$ -proteobacterium according to claim 5, wherein the *arcA* gene is DNA defined in the following (a) or (b):

(a) DNA containing the nucleotide sequence of the nucleotide numbers  
5 101 to 817 of SEQ ID NO: 17;

(b) DNA hybridizable with the nucleotide sequence of the nucleotide  
10 numbers 101 to 817 of SEQ ID NO: 17 or a probe that can be produced from  
the nucleotide sequence under the stringent condition and coding for a  
protein that improves an ability to produce a target substance when the  
protein does not normally function compared with the case where the protein  
normally functions.

[Claim 7] The  $\gamma$ -proteobacterium according to any one of claims 1 to 6,  
which is a bacterium belonging to the genus *Escherichia*.

[Claim 8] The  $\gamma$ -proteobacterium according to any one of claims 1 to 7,  
15 wherein the target substance is an L-amino acid.

[Claim 9] The  $\gamma$ -proteobacterium according to claim 8, wherein the L-amino  
acid is L-lysine or L-glutamic acid.

[Claim 10] A method for producing a target substance, which comprises  
culturing the  $\gamma$ -proteobacterium according to any one of claims 1 to 9  
20 in a medium to produce and accumulate the target substance in the medium  
or cells and collecting the target substance from the medium or cells.

[Detailed Description of the Invention]

[0001]

25 [Technical Field to which the Invention Pertains]

The present invention relates to a technique used in the fermentation  
industry, more precisely, a method for efficiently producing a target  
substance such as L-amino acids by fermentation utilizing a microorganism.

[0002]

30 [Prior Art]

Bacterial cells have been modifying their metabolic pathways, respiratory pathways and so forth in order to adapt to various environments.

In the energy metabolism, Arc (aerobic respiration control) and Fnr (fumarate nitrate reduction) are known as control systems playing important roles. These consist of global regulator proteins and universally existing in *E. coli* and other analogous species. The former is encoded by the *arcA* gene existing at the position of 0 minute of the *E. coli* chromosome, the latter is encoded by the *fnr* gene existing at the position of 29 minutes of the *E. coli* chromosome, and the both adapt the cell to an environment by controlling many factors under an anaerobic condition. Moreover, it has been elucidated that the ArcA protein and the Fnr protein are transcription factors, and they positively or negatively control expression of a target gene on the *E. coli* chromosome under an anaerobic condition by directly binding to a promoter region of the target gene (S. Iuchi et al., Cell, 66, 5-7 (1991)).

[0003]

Recently, expression profiles of strains in which genes coding global regulators such as the ArcA protein and Fnr protein derived from *E. coli* are disrupted are collected in a database by using DNA microarray techniques and opened to the public

([http://www.genome.ad.jp/dbget-bin/get\\_htext?Exp\\_DB+-n+Bget-bin/get\\_htext?Exp\\_DB+-n+B](http://www.genome.ad.jp/dbget-bin/get_htext?Exp_DB+-n+Bget-bin/get_htext?Exp_DB+-n+B)).

[0004]

So far, it is known that the ArcA protein negatively controls expression of the genes for the tricarboxylic acid cycle (S. Iuchi et al., Cell, 66, 5-7 (1991)), and the expression of the genes for the tricarboxylic acid cycle is increased in the *arcA*-disrupted strain in the database.

On the other hand, it is known that the Fnr protein positively controls gene expression for the respiratory pathway that functions under an anaerobic condition.

[0005]

As for the expression profiles in the global factor-disrupted strains, the *dam*-disrupted strain can be mentioned as a strain in which gene expression for the TCA is increased like the *arcA*-disrupted strain (H. Mori, Nara Institute of Science and Technology, oral announcement at the symposium "Green Biotechnology of Genome Age", 2001, organized by Japan Bioindustry Association, Resource Biotransformation Study Group).

[0006]

The Dam protein is a methylase for modification factors involved in intracellular restriction modification systems, and it is encoded by the *dam* gene existing at the position of 76 minutes of the *E. coli* chromosome (Proc. Natl. Acad. Sci. U.S.A., 87 (23), 9454-9458 (1990)).

[0007]

It has not been reported so far about improvement of substance production through expression control of the global factors such as genes *arcA*, *fnr* and *dam*.

[0008]

[Problem to be solved by the Invention]

An object of the present invention is to improve production efficiency in production of a useful substance by fermentation utilizing a  $\gamma$ -proteobacterium such as *Escherichia* bacteria.

[0009]

[Means to Solve the Problem]

The inventors of the present invention conducted various researches in order to achieve the aforementioned object, and they found production of substance by a  $\gamma$ -proteobacterium could be improved by modifying a gene coding for a regulator protein universally existing in  $\gamma$ -proteobacteria.

That is, they found that an ability to produce a target substance could be improved by disrupting the *arcA* gene in a  $\gamma$ -proteobacterium and thus accomplished the present invention.

[0010]

That is, the present invention provides followings.

(1) A  $\gamma$ -proteobacterium having an ability to produce a target substance and modified so that an ArcA protein does not normally function.

5 (2) The  $\gamma$ -proteobacterium according to (1), wherein the ArcA protein that normally functions is a protein defined in the following (A) or (B):

(A) a protein having the amino acid sequence of SEQ ID NO: 18;

(B) a protein having the amino acid sequence of SEQ ID NO: 18 including substitution, deletion, insertion or addition of one or several amino  
10 acids and improving an ability to produce a target substance when the protein does not normally function in the  $\gamma$ -proteobacterium compared with the case where the protein normally functions.

(3) The  $\gamma$ -proteobacterium according to (1), wherein the ArcA protein that normally functions is a protein having 70% or more of homology to the  
15 amino acid sequence of SEQ ID NO: 18 and improving an ability to produce a target substance when the protein does not normally function in the  $\gamma$ -proteobacterium compared with the case where the protein normally functions.

(4) The  $\gamma$ -proteobacterium according to (1), wherein the ArcA protein that  
20 normally functions is a protein having the amino acid sequence of SEQ ID NO: 18 including substitution, deletion, insertion or addition of 2 to 20 amino acids and improving an ability to produce a target substance when the protein does not normally function in the  $\gamma$ -proteobacterium compared with the case where the protein normally functions.

25 (5) The  $\gamma$ -proteobacterium according to any one of (1) to (4), wherein the ArcA protein does not normally function by means of disruption of an *arcA* gene on a chromosome.

(6) The  $\gamma$ -proteobacterium according to (5), wherein the *arcA* gene is DNA defined in the following (a) or (b):

30 (a) DNA containing the nucleotide sequence of the nucleotide numbers

101 to 817 of SEQ ID NO: 17;

(b) DNA hybridizable with the nucleotide sequence of the nucleotide numbers 101 to 817 of SEQ ID NO: 17 or a probe that can be produced from the nucleotide sequence under the stringent condition and coding for a protein that improves an ability to produce a target substance when the protein does not normally function compared with the case where the protein normally functions.

(7) The  $\gamma$ -proteobacterium according to any one of (1) to (6), which is a bacterium belonging to the genus *Escherichia*.

(8) The  $\gamma$ -proteobacterium according to any one of (1) to (7), wherein the target substance is an L-amino acid.

(9) The  $\gamma$ -proteobacterium according to (8), wherein the L-amino acid is selected from the group consisting of L-lysine, L-glutamic acid and L-arginine.

(10) A method for producing a target substance, which comprises culturing the  $\gamma$ -proteobacterium according to any one of (1) to (9) in a medium to produce and accumulate the target substance in the medium or cells and collecting the target substance from the medium or cells.

[0011]

[Embodiment of the Invention]

Hereafter, the present invention will be explained in detail.

[0012]

<1>  $\gamma$ -Proteobacterium of the present invention

The  $\gamma$ -proteobacterium used for the present invention is not particularly limited so long as it is a microorganism belonging to  $\gamma$ -proteobacteria such as genus *Escherichia*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, *Erwinia*, *Salmonella*, *Morganella* or the like and has an ability to produce a target substance. Specifically, those classified into the  $\gamma$ -proteobacteria according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database



(<http://www.ncbi.nlm.nih.gov/htbin-post/Taxonomy/wgetorg?mode=Tree&id=1236&lvl=3&keep=1&srchmode=1&unlock>) can be used.

[0013]

Examples of the bacterium belonging to the genus *Escherichia* include *E. coli* and so forth. Examples of the belonging to the genus *Enterobacter* include *Enterobacter agglomerans*, *Enterobacter aerogenes* and so forth.

There are some species of *Enterobacter agglomerans* recently re-classified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii agglomerans* or the like based on nucleotide sequence analysis of 16S rRNA etc. In the present invention, the bacterium may belong to either the genus *Enterobacter* or *Pantoea* so long as it is classified into  $\gamma$ -proteobacteria and has the *arcA* gene.

[0014]

When *E. coli* is bred by using genetic engineering techniques, the *E. coli* K12 strain and derivatives thereof can be used. Further, when *Pantoea ananatis* is bred by using genetic engineering techniques, *Pantoea ananatis* strains AJ13355 (FERM BP-6614), AJ13356 (FERM BP-6615) and AJ13601 (FERM BP-7207), and derivatives thereof can be used. Although the above-mentioned strains were identified as *Enterobacter agglomerans* when they were isolated, these strains has been re-classified into *Pantoea ananatis* based on nucleotide sequence analysis of 16S rRNA etc. as described above.

[0015]

The  $\gamma$ -proteobacterium of the present invention is any one of the aforementioned bacteria, and is a bacterium having an ability to produce a target substance. The "ability to produce a target substance" means an ability to produce and accumulate the target substance in cells or a medium in such a degree that, when the bacterium of the present invention is cultured in the medium, the target substance can be collected from the cells or medium.

The target substance to be produced according to the present invention is not particularly limited, so long as it is a substance that is produced by a  $\gamma$ -proteobacterium and synthesized via the tricarboxylic acid cycle or a substance synthesized from such a substance as a substrate. Examples include, for example, those conventionally produced by  $\gamma$ -proteobacteria, i.e., various amino acids such as L-lysine, L-threonine, L-isoleucine, L-glutamic acid, L-glutamine and L-arginine, organic acids such as L-homoserine and succinic acid and so forth. Further, the present invention can also be applied to a substance that has not so far been industrially produced by using  $\gamma$ -proteobacteria, so long as it can be synthesized from a substance synthesized via the TCA cycle as a substrate.

[0016]

As L-lysine producing  $\gamma$ -proteobacteria, there can be exemplified mutants having resistance to an L-lysine analogue. This L-lysine analogue is a substance that inhibits growth of L-amino acid producing strain, but this inhibition is fully or partially canceled when L-lysine coexists in a medium. Examples of the L-lysine analogue include oxalysine, lysine hydroxamate, S-(2-aminoethyl)-L-cysteine (AEC),  $\gamma$ -methyllysine,  $\alpha$ -chlorocaprolactam and so forth. Mutants having resistance to these lysine analogues can be obtained by subjecting  $\gamma$ -proteobacteria to a conventional artificial mutagenesis treatment. Specific examples of bacterial strain used for producing L-lysine include *E. coli* AJ11442 (FERM BP-1543, NRRL B-12185; refer to Japanese Patent Laid-open Publication (Kokai) No. 56-18596 and U.S. Patent No. 4,346,170) and *E. coli* VL611.

In these microorganisms, feedback inhibition of aspartokinase by L-lysine is desensitized.

[0017]

In addition to the above, there can be mentioned, for example, L-threonine producing bacteria described later, because inhibition of aspartokinase by L-lysine is generally eliminated also in L-threonine

producing bacteria.

[0018]

In the Examples described later, the WC196 strain was used as an L-lysine producing bacterium of *E. coli*. This bacterial strain was bred  
5 by imparting AEC resistance to the W3110 strain derived from *E. coli* K-12.

This strain was designated as the *E. coli* AJ13069, and was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (presently, the independent administrative corporation, International Patent Organism Depositary,  
10 National Institute of Advanced Industrial Science and Technology, postal code: 305-8566, Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on December 6, 1994 and received an accession number of FERM P-14690.

Then, it was converted to an international deposit under the provisions of the Budapest Treaty on September 29, 1995, and received an accession  
15 number of FERM BP-5252 (refer to International Patent Publication WO96/17930).

[0019]

Examples of L-threonine producing  $\gamma$ -proteobacteria include *E. coli* VKPM B-3996 (RIA 1867, refer to U.S. Patent No. 5,175,107), strain MG442  
20 (refer to Gusyatiner et al., Genetika (in Russian), 14, pp.947-956, 1978) and so forth.

[0020]

Examples of the microorganism belonging to  $\gamma$ -proteobacteria and having L-glutamic acid producing ability include, for example,  
25 microorganisms deficient in  $\alpha$ -ketoglutarate dehydrogenase activity or having reduced  $\alpha$ -ketoglutarate dehydrogenase activity. Bacteria belonging to the genus *Escherichia* deficient in  $\alpha$ -ketoglutarate dehydrogenase activity or having reduced  $\alpha$ -ketoglutarate dehydrogenase activity and methods for obtaining them are described in Japanese Patent  
30 Laid-open Publication (Kokai) Nos. 5-244970 and 7-203980. Specifically,

the following strains can be mentioned.

[0021]

*E. coli* W3110sucA::Km<sup>r</sup>

*E. coli* AJ12624 (FERM BP-3853)

5 *E. coli* AJ12628 (FERM BP-3854)

*E. coli* AJ12949 (FERM BP-4881)

[0022]

10 *E. coli* W3110sucA::Km<sup>r</sup> is a strain obtained by disrupting the  
α-ketoglutarate dehydrogenase gene (hereinafter referred to as "sucA gene")  
of *E. coli* W3110, and it is a strain completely deficient in the  
α-ketoglutarate dehydrogenase.

[0023]

15 Microorganisms belonging to γ-proteobacteria and deficient in  
α-ketoglutarate dehydrogenase activity or having reduced α-ketoglutarate  
dehydrogenase activity and methods for obtaining them are described in  
Japanese Patent Laid-open Publication (Kokai) Nos. 5-244970 and 7-203980.

[0024]

20 Examples of L-isoleucine producing γ-proteobacteria include *E. coli*  
KX141 (VKPM B-4781, refer to European Patent Laid-open Publication No.  
519,113).

[0025]

25 Examples of L-homoserine producing *Escherichia* bacteria include  
the NZ10 strain, which is a Leu<sup>+</sup> revertant of the C600 strain (refer to  
Appleyard R.K., Genetics, 39, pp.440-452, 1954).

[0026]

As succinic acid producing γ-proteobacteria, examples using *E. coli*  
are known (Wang, X., et al., Appl. Biochem. Biotech., 70-72, 919-928 (1998)).

[0027]

30 Further, bacteria belonging to the genus *Escherichia* having L-amino  
acid producing ability can also be bred by introducing DNA having genetic

information involved in biosynthesis of L-amino acids and enhancing the ability utilizing a gene recombination technique. For example, as for L-lysine producing bacteria, examples of genes that can be introduced include, for example, genes coding for enzymes of the biosynthetic pathway of L-lysine such as phosphoenolpyruvate carboxylase, aspartokinase, dihydrodipicolinate synthetase, dihydrodipicolinate reductase, succinyldiaminopimelate transaminase and succinyldiaminopimelate deacylase. In case of a gene of an enzyme suffering from feedback inhibition by L-aspartic acid or L-lysine such as phosphoenolpyruvate carboxylase or aspartokinase and dihydrodipicolinate synthetase, it is desirable to use a mutant gene coding for an enzyme in which such inhibition is eliminated.

[0028]

Further, as for L-glutamic acid producing bacteria, examples of genes that can be introduced include genes of glutamate dehydrogenase, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase, phosphoenolpyruvate carboxylase, pyruvate dehydrogenase, pyruvate kinase, phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase, fructose bis-phosphate aldolase, phosphofructokinase, glucose phosphate isomerase and so forth.

[0029]

Further, an activity of an enzyme that catalyzes a reaction for producing a compound other than the target L-amino acid by branching off from the biosynthetic pathway of the L-amino acid may be decreased or made deficient. For example, examples of such an enzyme that catalyzes a reaction for producing a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine include homoserine dehydrogenase (refer to International Patent Publication WO95/23864). Further, examples of an enzyme that catalyzes a reaction for producing a compound other

than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid include  $\alpha$ -ketoglutarate dehydrogenase, isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrophosphate dehydrogenase and so forth.

[0030]

In breeding of  $\gamma$ -proteobacteria having such a target substance producing ability as mentioned above, to introduce a gene into  $\gamma$ -proteobacteria to enhance their ability, there can be used a method in which a vector autonomously replicable in a  $\gamma$ -proteobacterium cell is ligated to the gene to produce recombinant DNA and  $\gamma$ -proteobacterium is transformed with it. In addition, it is also possible to incorporate a target gene into host chromosome by a method using transduction, transposon (Berg, D.E. and Berg, C.M., Bio/Technol. 1, p.417, 1983), Mu phage, (Japanese Patent Laid-open Publication (Kokai) No. 2-109985) or homologous recombination (Experiments in Molecular Genetics, Cold Spring Harbor Lab., 1972). Further, the target gene can also be introduced by a method of disrupting a gene using a linear DNA produced by PCR (Kirill A., Datsenko et al., Proc. Natl. Acad. Sci. USA., 97 (12), 6640-6645 (2000)).

[0031]

Examples of the  $\gamma$ -proteobacteria bred by recombinant DNA techniques as described above include, for example, bacteria belonging to the genus *Escherichia* having enhanced activities of dihydrodipicolinate synthase having a mutation canceling feedback inhibition by L-lysine, aspartokinase, dihydrodipicolinate reductase and so forth, of which feedback inhibition by L-lysine is desensitized, and having L-lysine producing ability (U.S. Patent No. 6,040,160), and bacterium belonging to the genus *Enterobacter* (the genus *Pantoea*) having enhanced activity of citrate synthase, phosphoenolpyruvate carboxylase or glutamate dehydrogenase and having L-glutamic acid producing ability (EP 0 952 221 A2, EP 0 999 282 A2, EP

1 078 989 A2).

[0032]

The  $\gamma$ -proteobacterium used for the present invention is a bacterium having an ability to produce the aforementioned target substance and modified so that the ArcA protein does not normally function in a cell. The expression of "modified so that the ArcA protein does not normally function in a cell" means that it is modified so that the function of the ArcA protein should be completely eliminated, or the function should be reduced compared with an unmodified strain of *Escherichia* bacterium such as a wild strain.

The state where the ArcA protein does not normally function may be, for example, a state where transcription or translation of the *arcA* gene is inhibited, and hence the gene product thereof, the ArcA protein, is not produced or the production thereof is reduced, or a state where the produced ArcA protein is mutated, and thus the proper function of the ArcA protein is reduced or eliminated. Examples of the  $\gamma$ -proteobacteria in which the ArcA protein does not normally function include, typically, a gene-disrupted strain in which the *arcA* gene on the chromosome is disrupted by a genetic recombination technique, and a mutant strain in which an expression regulatory sequence or coding region of the *arcA* gene on the chromosome is mutated, and therefore functional ArcA protein is no longer produced.

[0033]

Examples of the ArcA protein contained in a wild strain or unmodified strain used for the breeding of the bacterium of the present invention include, for example, a protein having the amino acid sequence of SEQ ID NO: 18. Further, examples of the *arcA* gene include, for example, DNA having the nucleotide sequence of SEQ ID NO: 17. Moreover, the gene may have the sequence in which any codon is replaced with another equivalent codon. In the present invention, the term "DNA coding for a protein" means that, when DNA is double-stranded, either one of the strands codes for the protein.

[0034]

Further, the ArcA protein contained in the wild strain or unmodified strain is not limited to a wild-type protein, and it may contain substitution, deletion, insertion, addition or the like of one or more amino acid residues so long as the protein has the activity of ArcA protein. Although the number of "several" amino acid residues referred to herein differs depending on position or type of amino acid residues in the three-dimensional structure of the protein, it may be specifically 2 to 30, preferably 2 to 20, more preferably 2 to 10.

[0035]

The aforementioned "activity of the ArcA protein" is an activity that improves the ability to produce a target substance when the protein does not function normally compared with the case where the protein normally functions. In other words, the activity of the ArcA protein means that a  $\gamma$ -proteobacterium modified so that the protein does not normally function produces and accumulates a larger amount of the target substance in a medium compared with an unmodified strain of the  $\gamma$ -proteobacterium such as a wild strain. Examples of wild strain of *E. coli* include, for example, the K12 strain and derivative thereof such as *E. coli* MG1655 strain (ATCC No. 47076) and W3110 strain (ATCC No. 27325). Further, examples of unmodified strain of *Pantoea ananatis* (*Enterobacter agglomerans*) include the strains AJ13355 (FERM BP-6614), AJ13356 (FERM BP-6615) and AJ13601 (FERM BP-7207).

[0036]

The aforementioned substitution, deletion, insertion, addition, inversion or the like of amino acid residues also include naturally occurring mutations or variations due to difference in individual, species, strain or the like of the microorganism containing the ArcA protein.

[0037]

Examples of such mutants or variants of the *arcA* gene as mentioned



above include DNA that is hybridizable with a nucleotide sequence comprising the sequence of the nucleotide numbers 101 to 817 in SEQ ID NO: 17 or a probe that can be produced from the nucleotide sequence under the stringent condition and codes for a protein having an activity similar to that of ArcA. The "stringent condition" used herein is a condition under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent condition is exemplified by a condition under which DNAs having high homology, for example, DNAs having homology of 50% or more, preferably 70% or more, more preferably 80% or more, are hybridized with each other, but DNAs having homology lower than the above are not hybridized with each other. More specifically, the stringent condition is exemplified by a condition under which DNAs are hybridized with each other at a salt concentration corresponding to an ordinary condition of washing in Southern hybridization, i.e., 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C.

[0038]

As the probe, a partial sequence of the nucleotide sequence of SEQ ID NO: 17 can also be used. Such a probe can be prepared by PCR using oligonucleotides produced based on the nucleotide sequence of SEQ ID NO: 17 as primers and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 17 as a template. When a DNA fragment having a length of about 300 bps is used as the probe, the washing conditions for the hybridization may consist of 50°C, 2 x SSC and 0.1% SDS.

[0039]

The terms *arcA* gene and ArcA protein used hereafter are not limited to those having the nucleotide sequence or amino acid sequence shown in SEQ ID NO: 17 or 18, but include mutants or homologues thereof.

[0040]

The bacterium of the present invention is a bacterium modified

so that the ArcA protein does not normally function, specifically, a  $\gamma$ -proteobacterium of which *arcA* gene is disrupted, for example. Such a bacterium can be obtained by, for example, substituting an *arcA* gene that does not normally function (hereafter also referred to as "disrupted *arcA* gene") for the *arcA* gene on the chromosome by homologous recombination utilizing a genetic recombination technique (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press (1972); Matsuyama, S. and Mizushima, S., J. Bacteriol., 162, 1196 (1985)).

[0041]

The mechanism of the homologous recombination is as follows. When a plasmid or the like carrying a sequence exhibiting homology with a chromosomal sequence is introduced into a corresponding bacterial cell, recombination occurs at a site of the homologous sequence at a certain frequency, and thus the introduced plasmid as a whole is integrated into the chromosome. Then, by causing recombination again at the site of the homologous sequence on the chromosome, the plasmid may be removed again from the chromosome. However, depending on the position at which the recombination is caused, the disrupted gene may remain on the chromosome, while the original normal gene may be removed from the chromosome together with the plasmid. By selecting such a bacterial strain, a bacterial strain in which the normal *arcA* gene is replaced with the disrupted *arcA* gene can be obtained.

[0042]

Such a gene disruption technique based on the homologous recombination has already been established, and a method utilizing a linear DNA, a method utilizing temperature sensitive plasmid or the like can be used therefor. The *arcA* gene can also be disrupted by using a plasmid that contains the *arcA* gene inserted with a marker gene such as drug resistance gene, and cannot replicate in a target microbial cell. That is, in a transformant that has been transformed with such a plasmid and hence acquired

drug resistance, the marker gene is integrated into the chromosome DNA.

It is likely that this marker gene has been integrated by homologous recombination of the *arcA* gene present at the both sides of the marker with these genes on the chromosome, and therefore a gene-disrupted strain can efficiently be selected.

[0043]

Examples of temperature sensitive plasmid functioning in *Escherichia* bacteria include pMAN997 (International Patent Publication WO99/03988), pHSG415, pHSG422 (Hashimoto-Gotoh, T. et al, Gene, 16, 227-235 (1981)) and so forth.

[0044]

Specifically, a disrupted *arcA* gene used for the gene disruption can be obtained by deletion of a certain region of *arcA* gene by means of digestion with restriction enzyme(s) and religation, by insertion of another DNA fragment (marker gene etc.) into the *arcA* gene, or by introducing substitution, deletion, insertion, addition or inversion of one or more nucleotides in a nucleotide sequence of coding region of *arcA* gene, its promoter region or the like by means of site-specific mutagenesis (Kramer, W. and Frits, H. J., Methods in Enzymology, 154, 350 (1987)) or treatment with a chemical reagent such as sodium hyposulfite and hydroxylamine (Shortle, D. and Nathans, D., Proc. Natl. Acad. Sci. U.S.A., 75, 270 (1978)) or the like, so that the activity of the encoded repressor should be reduced or eliminated, or transcription of the *arcA* gene should be reduced or eliminated. Among these methods, a method utilizing deletion of a certain region of the *arcA* gene by digestion with a restriction enzyme and religation, or insertion of another DNA fragment into the *arcA* gene is preferred in view of reliability and stability.

[0045]

The sequence of *arcA* gene per se is known, and therefore the *arcA* gene can be easily obtained by the PCR method or hybridization method

based on the sequence. It is sufficient that the *arcA* gene used for the gene disruption should have homology in such a degree that homologous recombination with the *arcA* gene contained in the target bacterium should be caused. Specifically, it is sufficient that the homology should be usually 70% or more, preferably 80% or more, more preferably 90% or more. [0046]

Disruption of the target gene can be confirmed by analyzing the gene on the chromosome utilizing Southern blotting or PCR method.

[0047]

Methods for obtaining various genes, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, transformation etc. used for the present invention are described in Sambrook, J., Fritsch, E.F., Maniatis, T., Molecular Cloning, Cold Spring Harbor Laboratory Press, 1.21 (1989).

[0048]

Further, a mutant strain in which functional ArcA protein is no longer produced can be obtained by subjecting a  $\gamma$ -proteobacterium to ultraviolet irradiation or treating it with a mutating agent used for usual mutation treatment such as N-methyl-N'-nitrosoguanidine (NTG) or nitrous acid.

[0049]

By culturing a  $\gamma$ -proteobacterium microorganism having an ability to produce a target substance and modified so that the ArcA protein does not normally function, which can be obtained as described above, in a medium to produce and accumulate the target substance in the medium or cells and collecting the target substance from the medium or cells, the target substance can be produced. According to the present invention, the production efficiency of the target substance can be improved by using a  $\gamma$ -proteobacterium having the aforementioned characteristics. It is estimated that the *arcA* gene is expressed in a wild strain of  $\gamma$ -proteobacterium

concerning the *arcA* gene during the culture and inhibits the expression of the genes involved in the TCA cycle, whereas in a strain in which the ArcA protein does not normally function, such expression inhibition for the TCA cycle genes is canceled, and thus the above effect should be obtained.

5 [0050]

The medium used for the present invention may be an ordinary medium containing a carbon source, nitrogen source, inorganic ions, and other organic components as required. As the carbon source, there can be used saccharides such as glucose, lactose, galactose, fructose, arabinose, maltose, xylose, trehalose, ribose and starch hydrolysate, alcohols such as glycerol, mannitol and sorbitol and organic acids such as gluconic acid, fumaric acid, citric acid and succinic acid. As the nitrogen source, there can be used inorganic ammonium salts such as ammonium sulfate, ammonium chloride and ammonium phosphate, organic nitrogen such as soybean protein hydrolysate, ammonia gas, aqueous ammonia and so forth. As organic trace amount nutrients, it is desirable to add required substances, for example, vitamins such as vitamin B<sub>1</sub>, nucleic acids such as adenine and RNA or yeast extract or the like to the medium in appropriate amounts. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and so forth are added in small amounts as required.

10  
15  
20

[0051]

The culture may be carried out under conventionally used well-known conditions depending on the bacterial strain used. For example, the culture is preferably carried out under an aerobic condition for 16 to 72 hours.

Culture temperature is preferably controlled to be 30°C to 45°C, and pH is preferably controlled to be 4.5 to 8 during the culture. Inorganic or organic, acidic or alkaline substances as well as ammonia gas and so forth can be used for pH adjustment.

25

[0052]

30 For collection of the target substance from the medium or cells,

any special method is not required for the present invention. That is, it can be carried out by a combination of conventionally well-known techniques such as methods utilizing ion exchange resins, precipitation and other techniques depending on the type of the target substance. Further, the target substance accumulated in cells can be collected, after the cells are physically or enzymatically disrupted, from cell extract or membrane fraction depending on the target substance. Furthermore, depending on the target substance, cells containing the target substance can also be used as they are as a microbial catalyst or the like.

[0053]

[Examples]

Hereafter, the present invention will be explained more specifically with reference to the following examples.

[0054]

Example 1: Disruption of *arcA*, *dam* and *fnr* genes of *E. coli*

The entire nucleotide sequence of genomic DNA of *E. coli* K-12 strain has been already elucidated (Blattner F.R., Plunkett G., Bloch C.A. et al., Science, 227, 1453-1474 (1997);

<ftp://ftp.genetics.wisc.edu/pub/sequence/ecolim52.seq.gz>). Based on

the known nucleotide sequences of *arcA*, *dam* and *fnr* genes, gene-disrupted strains for each of *arcA*, *dam* and *fnr* were produced. In the following procedure, QIAGEN-Genomic-tip System (produced by QIAGEN) was used for the extraction of genomic DNA.

[0055]

(1) Disruption of *arcA* gene of *E. coli*

Primers were synthesized based on the reported nucleotide sequence of *arcA*, and N- and C-terminal fragments of *arcA* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template.

Pyrobest DNA Polymerase (produced by Takara Shuzo) was used for PCR, and PCR was performed according to the attached instruction. Primers

1 and 2 were used as the primers for PCR for amplifying N-terminal fragment, and Primers 3 and 4 were used as the primers for PCR for amplifying C-terminal fragment. Primer 1 was designed to contain a *Hind*III site, and Primer 4 was designed to contain an *Xba*I site.

5 [0056]

Primer 1: cccaagcttaaagccctttacttagctta (sequence complementary to the nucleotide numbers 5482 to 5501 of the nucleotide sequence of GenBank Accession No. AE000510 added with ccc and *Hind*III site at the 5' end, SEQ ID NO: 1)

10 Primer 2: tccgcgccatctgtcgcttc (sequence of the nucleotide numbers 4851 to 4870 of the nucleotide sequence of GenBank Accession No. AE000510, SEQ ID NO: 2)

Primer 3: gaagcgacagatggcgcggaagctacaagttcaatggt (sequence complementary to the nucleotide numbers 4541 to 4560 of the nucleotide sequence of GenBank Accession No. AE000510 added at the 5' end with a sequence complementary to the nucleotide numbers 4851 to 4870 of the nucleotide sequence of GenBank Accession No. AE000510, SEQ ID NO: 3)

15 Primer 4: gggctctagagggttgaaaaataaaaacggc (sequence of the nucleotide numbers 4188 to 4207 of the nucleotide sequence of GenBank Accession No. AE000510 added with ggg and *Xba*I site at the 5' end, SEQ ID NO: 4)

20 [0057]

After PCR, the amplified DNA fragments were each purified by using QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal DNA fragment and C-terminal DNA fragment, Primers 1 and 4 were used for the crossover PCR method (A.J. Link, D. Phillips, G.M. Church, Journal of Bacteriology, 179, 6228-6237 (1997)) to obtain a disrupted *arcA* fragment.

25 The purified DNA fragment was digested with *Hind*III and *Xba*I (produced by Takara Shuzo) and subjected to phenol/chloroform treatment and ethanol precipitation. This fragment was ligated with a temperature sensitive plasmid pMAN997 (International Patent Publication WO99/03988) also

30

digested with *Hind*III and *Xba*I by using DNA ligation Kit Ver.2 (produced by Takara Shuzo). JM109 competent cells (produced by Takara Shuzo) were transformed with this ligation solution and applied to an LB agar plate containing 25 µg/mL of ampicillin (produced by Sigma) (LB + ampicillin plate). After the cells were cultured at 30°C for one day, the grown colonies were cultured in test tubes at 30°C in LB medium containing 25 µg/mL of ampicillin, and plasmids were extracted by using an automatic plasmid extractor PI-50 (produced by Kurabo Industries). The obtained plasmids were digested with *Hind*III and *Xba*I and subjected to agarose gel electrophoresis, and the plasmid inserted with the target fragment was designated as plasmid pMAN\_ΔarcA for *arcA* disruption. The aforementioned pMAN997 is a plasmid obtained by exchanging *Vsp*I-*Hind*III fragments of pMAN031 (S. Matsuyama and S. Mizushima, J. Bacteriol., 162, 1196 (1985)) and pUC19 (produced by Takara Shuzo).

[0058]

The *E. coli* WC196 strain was transformed with the plasmid pMAN\_ΔarcA according to the method of C.T. Chung et al., and colonies were selected on an LB + ampicillin plate at 30°C. The selected clones were cultured overnight at 30°C as liquid culture, then the culture broth was diluted to 10<sup>-3</sup> concentration and plated on an LB + ampicillin plate, and colonies were selected at 42°C. The selected clones were applied to an LB + ampicillin plate and cultured at 30°C, and then 1/8 of the cells on the plate were suspended in 2 mL of LB medium and cultured at 42°C for 4 to 5 hours with shaking. The culture broth was diluted to 10<sup>-5</sup> concentration and applied to an LB plate, and several hundreds of colonies among the obtained colonies were inoculated on an LB plate and LB + ampicillin plate to confirm growth and thereby select ampicillin sensitive strains. Colony PCR was performed for several ampicillin sensitive strains to confirm the deletion of *arcA* gene. In this way, an *arcA* disrupted-strain derived from *E. coli* WC196, WC196ΔarcA, was obtained.



[0059]

(2) Disruption of *dam* gene of *E. coli*

A *dam* gene-disrupted strain was produced from WC196 in the same manner as in (1).

5           Primers were synthesized based on the reported nucleotide sequence of the *dam* gene, and N- and C-terminal fragments of the *dam* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template. Primers 5 and 6 were used as the primers for PCR for amplifying N-terminal fragment, and Primers 7 and 8 were used as the primers for  
10          PCR for amplifying C-terminal fragment. Primer 5 was designed to contain a *Hind*III site, and Primer 8 was designed to contain an *Xba*I site.

[0060]

Primer 5: cccaagcttccgtggtatgtccttggttc (sequence complementary to the nucleotide numbers 5150 to 5169 of the nucleotide sequence of GenBank  
15          Accession No. AE000414 added with ccc and *Hind*III site at the 5' end, SEQ ID NO: 5)

Primer 6: agactgatcaggtcgctatt (sequence of the nucleotide numbers 4741 to 4760 of the nucleotide sequence of GenBank Accession No. AE000414, SEQ ID NO: 6)

20          Primer 7: aatagcgacctgatcagtctgccttatgcaccgctgtctg (sequence complementary to the nucleotide numbers 4361 to 4380 of the nucleotide sequence of GenBank Accession No. AE000414 added at the 5' end with a sequence complementary to the nucleotide numbers 4741 to 4760 of the nucleotide sequence of GenBank Accession No. AE000414, SEQ ID NO: 7) Primer  
25          8: gggcttagacgtcagattgggaacatagt (sequence of the nucleotide numbers 3931 to 3950 of the nucleotide sequence of GenBank Accession No. AE000414 added with ggg and *Xba*I site at the 5' end, SEQ ID NO: 8)

[0061]

30          After PCR, the amplified DNA fragments were each purified by using QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal

DNA fragment and C-terminal DNA fragment, Primers 5 and 8 were used for the crossover PCR method to obtain a deficient type *dam* fragment. The following procedure was performed in the same manner as in (1) to obtain a *dam* disrupted-strain WC196 $\Delta$ dam.

[0062]

### (3) Disruption of *fnr* gene of *E. coli*

A *fnr* gene-disrupted strain was produced from WC196 in the same manner as in (1).

Primers were synthesized based on the reported nucleotide sequence of the *fnr* gene, and N- and C-terminal fragments of the *fnr* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template.

Primers 9 and 10 were used as the primers for PCR for amplifying N-terminal fragment, and Primers 11 and 12 were used as the primers for PCR for amplifying C-terminal fragment. Primer 9 was designed to contain a *Hind*III site, and Primer 12 was designed to contain an *Xba*I site. A *fnr*-disrupted strain was produced from WC196 in the same manner as in (1).

[0063]

Primer 9: cccaagcttgcaattgggccgtcctggcg (sequence complementary to the nucleotide numbers 7981 to 8000 of the nucleotide sequence of GenBank Accession No. AE000231 added with ccc and *Hind*III site at the 5' end, SEQ ID NO: 9)

Primer 10: tcaagctgatcaagctcatg (sequence of the nucleotide numbers 7501 to 7520 of the nucleotide sequence of GenBank Accession No. AE000231, SEQ ID NO: 10)

Primer 11: gaaaaatgcc gaggaacgtc tcaagctgat caagctcatg (sequence complementary to the nucleotide numbers 7121 to 7140 of the nucleotide sequence of GenBank Accession No. AE000231 added at the 5' end with a sequence complementary to the nucleotide numbers 7501 to 7520 of the

nucleotide sequence of GenBank Accession No. AE000231, SEQ ID NO: 11)  
 Primer 12: gggctctagattggctcgtcctggtaggat (sequence of the nucleotide  
 numbers 6671 to 6690 of the nucleotide sequence of GenBank Accession No.  
 AE000231 added with ggg and *Xba*I site at the 5' end, SEQ ID NO: 12)

5 [0064]

After PCR, the amplified DNA fragments were each purified by using  
 QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal  
 DNA fragment and C-terminal DNA fragment, Primers 9 and 12 were used for  
 the crossover PCR method to obtain a deficient type *dam* fragment. The  
 10 following procedure was performed in the same manner as in (1) to obtain  
 a *fnr* disrupted-strain WC196Δ*fnr*.

Example 2: Effect of *arcA* disruption on L-lysine production in *E. coli*  
 strain

15 The *arcA* gene-disrupted strain, WC196Δ*arcA* strain, the *dam*  
 gene-disrupted strain, WC196Δ*dam*, the *fnr* gene-disrupted strain, WC196Δ*fnr*,  
 and the parent strain thereof, WC196, were cultured, and their L-lysine  
 production amounts were measured. The media, culture methods and analysis  
 method for the measurement are shown below.

20 [0065]

[Base medium: E-100 medium]

	Final concentration
Glucose	10 g/L (separately sterilized)
NH <sub>4</sub> Cl	20 mM
25 NaHPO <sub>4</sub>	40 mM
KH <sub>2</sub> PO <sub>4</sub>	30 mM
CaCl <sub>2</sub>	0.01 mM
FeSO <sub>4</sub>	0.01 mM
MnSO <sub>4</sub>	0.01 mM
30 citric acid	5 mM

thiamine hydrochloride 2 mM (separately sterilized)  
casamino acid 2.5 g/L (separately sterilized)  
MES-NaOH (pH 6.8) 50 mM (separately sterilized)

[0066]

5 [Culture method]

Refresh culture:

Stock bacteria were inoculated.

LB agar medium (drug was added as required), 37°C, 24 hours.

Seed culture:

10 The bacteria undergone the refresh culture were inoculated in a  
volume of 2 mL to LB medium.

LB medium (drug was added as required), 37°C, overnight.

Main culture:

1/16 of the bacteria on the seed culture cell plate were inoculated.

15 E-100 medium (drug was added as required), 37°C, 20 ml in 500 ml-volume  
Sakaguchi flask.

[0067]

[Analysis method]

20 The culture broth was sampled in a volume of 500 µl in a time course,  
and glucose concentration and L-lysine accumulation in the culture broth  
were measured. The glucose concentration and L-lysine accumulation were  
measured for supernatant of the culture broth obtained after centrifugation  
at 15,000 rpm for 5 minutes diluted to an appropriate concentration with  
water by using Biotech Analyzer (Sakura Seiki). The results are shown  
25 in Fig. 1.

[0068]

30 As a result, it was observed that the *fnr* gene-disrupted strain  
exhibited L-lysine accumulation equivalent to that of the control strain,  
and the *dam* gene-disrupted strain exhibited reduced accumulation compared  
with the control strain. On the other hand, it was recognized that the

L-lysine accumulation of the *arcA* gene-disrupted strain was improved compared with the control strain.

[0069]

Example 3: Effect of *arcA* disruption on L-glutamic acid production in *E. coli* strain

Since L-lysine accumulation improvement effect was observed in Example 2 by the use of *arcA* gene disruption, effect of the *arcA* gene on the L-glutamic acid fermentation was examined in this example.

In order to confirm effect of deficiency of the *arcA* gene on L-glutamic acid production in *E. coli* MG1655, *E. coli* MG1655-derived *sucA* deficient strain (MG1655 $\Delta$ *sucA*) and *E. coli* MG1655-derived *sucA* and *arcA* doubly deficient strain (MG1655 $\Delta$ *sucA* $\Delta$ *arcA*) were constructed.

[0070]

(1) Disruption of *sucA* gene of *E. coli*

A *sucA* gene-disrupted strain was produced from MG1655 in the same manner as in Example 1.

Primers were synthesized based on the reported nucleotide sequence of the *sucA* gene, and N- and C-terminal fragments of the *sucA* gene were amplified by PCR method using the genomic DNA of *E. coli* MG1655 strain as a template.

Primers 13 and 14 were used as the primers for PCR for amplifying N-terminal fragment, and Primers 15 and 16 were used as the primers for PCR for amplifying C-terminal fragment. Primer 13 was designed to contain a *HindIII* site, and Primer 16 was designed to contain an *XbaI* site. A *sucA*-disrupted strain was produced from MG1655 in the same manner as in (1).

[0071]

Primer 13: cccaagcttctgcccctgacactaagaca (sequence of the nucleotide numbers 10721 to 10740 of the nucleotide sequence of GenBank Accession No. AE000175 added with ccc and *HindIII* site at the 5' end, SEQ ID NO:

13)

Primer 14: cgaggtaacgttcaagacct (sequence complementary to the nucleotide numbers 11501 to 11520 of the nucleotide sequence of GenBank Accession No. AE000175, SEQ ID NO: 14)

5 Primer 15: aggtcttgaacgttacctcgatccataacgggcagggcgc (sequence of the nucleotide numbers 12801 to 12820 of the nucleotide sequence of GenBank Accession No. AE000175 added at the 5' end with a sequence of the nucleotide numbers 10501 to 11520 of the nucleotide sequence of GenBank Accession No. AE000175, SEQ ID NO: 15)

10 Primer 16: gggctctagaccactttgtcagtttcgatt (sequence complementary to the nucleotide numbers 13801 to 13820 of the nucleotide sequence of GenBank Accession No. AE000175 added with ggg and XbaI site at the 5' end, SEQ ID NO: 16)

[0072]

15 After PCR, the amplified DNA fragments were each purified by using QIAquick PCR Purification Kit (produced by QIAGEN). The purified N-terminal DNA fragment and C-terminal DNA fragment, Primers 13 and 16 were used for the crossover PCR method to obtain a deficient type *sucA* fragment. The following procedure was performed in the same manner as in (1) to  
20 obtain a *sucA* disrupted-strain, MG1655Δ*sucA*.

[0073]

(2) Preparation of *sucA* and *arcA* gene doubly deficient strain of *E. coli*

In the same manner as in Example 1, the *arcA* gene of MG1655Δ*sucA* was disrupted to prepare a *sucA* and *arcA* doubly deficient strain

25 (MG1655Δ*sucA*Δ*arcA*).

Similarly, *sucA* and *dam* doubly deficient strain (MG1655Δ*sucA*Δ*dam*) and *sucA* and *fnr* doubly deficient strain (MG1655Δ*sucA*Δ*fnr*) were produced.

[0074]

30 In order to examine effect of *arcA* gene disruption on L-glutamic acid fermentation, the doubly deficient strains for the genes,

MG1655 $\Delta$ sucA $\Delta$ arcA, MG1655 $\Delta$ sucA $\Delta$ dam, and MG1655 $\Delta$ sucA $\Delta$ fnr strains as well as the sucA gene deficient strain, MG1655 $\Delta$ sucA, as a control were cultured, and L-glutamic acid production amounts were measured. The media, culture methods and analysis method for the measurement are shown below.

5 [0075]

[Base medium: MS medium]

	Final concentration
Glucose	40 g/L (separately sterilized)
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1 g/L (separately sterilized)
10 (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	16 g/L
KH <sub>2</sub> PO <sub>4</sub>	1 g/L
Yeast extract	2 g/L
FeSO <sub>4</sub>	0.01 g/L
MnSO <sub>4</sub>	0.01 g/L
15 CaCO <sub>3</sub>	30 g/L (separately sterilized)

[0076]

[Culture methods]

Refresh culture:

Stock bacteria were inoculated.

20 LB agar medium (drug was added as required), 37°C, 24 hours.

Seed culture in test tube:

The bacteria undergone the refresh culture were inoculated.

LB liquid medium (drug was added as required), 37°C, 16 hours.

Main culture:

25 10% of the liquid medium for the seed culture was inoculated.

MS liquid medium (drug was added as required), 37°C, 20 ml in 500 ml-volume Sakaguchi flask.

[0077]

[Analysis method]

30 The culture broth was sampled in a volume of 500  $\mu$ l in a time course,

and glucose concentration and L-glutamic acid accumulation in the culture broth were measured. The glucose concentration and L-glutamic acid concentration were measured for supernatant of the culture broth obtained after centrifugation at 15,000 rpm for 5 minutes diluted to an appropriate concentration with water by using Biotech Analyzer (Sakura Seiki). The L-glutamic acid accumulation and yield at the point where the saccharide was depleted are shown in Table 1.

[0078]

[Table 1]

Table 1: L-glutamic acid accumulation and yield of *sucA* and *arcA*-disrupted strain

Strain	L-glutamic acid accumulation (g/L)	L-glutamic acid yield (%)
MG1655Δ <i>sucA</i>	15.4	36.9
MG1655Δ <i>sucA</i> Δ <i>arcA</i>	17.0	41.7
MG1655Δ <i>sucA</i> Δ <i>dam</i>	14.2	35.5
MG1655Δ <i>sucA</i> Δ <i>fnr</i>	14.6	36.6

[0079]

As a result, both of the accumulation and yield of glutamic acid were slightly lower in the *sucA* and *dam* gene-disrupted strain compared with the control, and they were comparable to those of the control in the *sucA* and *fnr* gene-disrupted strain. On the other hand, it was recognized that both of the accumulation and yield of L-glutamic acid were improved in the *sucA* and *arcA* gene-disrupted strain compared with the control strain.

[0080]

[Advantages of the invention]

According to the present invention, when a useful substance such as L-amino acids is produced by using a  $\gamma$ -proteobacterium, the production efficiency can be improved.



33  
SEQUENCE LISTING

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<120> Method for Producing Target Substance by Fermentation

<130> P-9999

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<141> 2002-07-12

[0082]

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[0083]

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amplifying Escherichia coli arcA gene

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20

[0084]

<210> 3

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<223> Description of Artificial Sequence: primer for  
sequencing of Escherichia coli arcA gene

<400> 3

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40

[0085]

<210> 4

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29

[0086]

<210> 5

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: primer for  
amplifying Escherichia coli dam gene

<400> 5

cccaagcttc cgtggtatgt cctggtttc

29

[0087]

<210> 6

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<212> DNA

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amplifying Escherichia coli dam gene

<400> 6

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20

[0088]

<210> 7

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sequencing of Escherichia coli dam gene

<400> 7

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40

[0089]

<210> 8

<211> 29

<212> DNA

<213> Artificial Sequence

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amplifying Escherichia coli dam gene

<400> 8

gggtctagac gtcagattgg gaacatagt

29

[0090]

<210> 9

<211> 29

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: primer for  
amplifying Escherichia coli fnr gene

<400> 9

cccaagcttg caattgggcc gtcctggcg

29

[0091]

<210> 10

<211> 20

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<223> Description of Artificial Sequence: primer for  
amplifying Escherichia coli fnr gene

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20

[0092]

<210> 11

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<212> DNA

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[0093]

<210> 12

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sequencing of Escherichia coli fnr gene

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[0094]

<210> 13

<211> 29

<212> DNA

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<400> 13

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29

[0095]

<210> 14

<211> 20

<212> DNA

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[0096]

<210> 15

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40

[0097]

&lt;210&gt; 16

&lt;211&gt; 29

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: primer for  
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&lt;400&gt; 16

gggtctagac cactttgtca gtttcgatt

29

[0098]

&lt;210&gt; 17

&lt;211&gt; 927

&lt;212&gt; DNA

&lt;213&gt; Escherichia coli

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (101).. (817)

&lt;400&gt; 17

gtcatgttac gccgatcatg ttaatttgca gcatgcatca ggcaggtcag ggacttttgt 60  
acttcctgtt tcgatttagt tggcaattta ggtagcaaac atg cag acc ccg cac 115

Met Gln Thr Pro His

1

5

att ctt atc gtt gaa gac gag ttg gta aca cgc aac acg ttg aaa agt 163  
Ile Leu Ile Val Glu Asp Glu Leu Val Thr Arg Asn Thr Leu Lys Ser

10

15

20

att ttc gaa gcg gaa ggc tat gat gtt ttc gaa gcg aca gat ggc gcg 211  
Ile Phe Glu Ala Glu Gly Tyr Asp Val Phe Glu Ala Thr Asp Gly Ala

25

30

35

gaa atg cat cag atc ctc tct gaa tat gac atc aac ctg gtg atc atg 259  
Glu Met His Gln Ile Leu Ser Glu Tyr Asp Ile Asn Leu Val Ile Met

40

45

50

gat atc aat ctg ccg ggt aag aac ggt ctt ctg tta gcg cgt gaa ctg 307  
Asp Ile Asn Leu Pro Gly Lys Asn Gly Leu Leu Leu Ala Arg Glu Leu

55

60

65

cgc gag cag gcg aat gtt gcg ttg atg ttc ctg act ggc cgt gac aac 355  
Arg Glu Gln Ala Asn Val Ala Leu Met Phe Leu Thr Gly Arg Asp Asn

39

70	75	80	85	
gaa gtc gat aaa att ctc ggc ctc gaa atc ggt gca gat gac tac atc				403
Glu Val Asp Lys Ile Leu Gly Leu Glu Ile Gly Ala Asp Asp Tyr Ile				
	90	95	100	
acc aaa ccg ttc aac ccg cgt gaa ctg acg att cgt gca cgc aac cta				451
Thr Lys Pro Phe Asn Pro Arg Glu Leu Thr Ile Arg Ala Arg Asn Leu				
	105	110	115	
ctg tcc cgt acc atg aat ctg ggt act gtc agc gaa gaa cgt cgt agc				499
Leu Ser Arg Thr Met Asn Leu Gly Thr Val Ser Glu Glu Arg Arg Ser				
	120	125	130	
gtt gaa agc tac aag ttc aat ggt tgg gaa ctg gac atc aac agc cgt				547
Val Glu Ser Tyr Lys Phe Asn Gly Trp Glu Leu Asp Ile Asn Ser Arg				
	135	140	145	
tcg ttg atc ggc cct gat ggc gag cag tac aag ctg ccg cgc agc gag				595
Ser Leu Ile Gly Pro Asp Gly Glu Gln Tyr Lys Leu Pro Arg Ser Glu				
	150	155	160	165
ttc cgc gcc atg ctt cac ttc tgt gaa aac cca ggc aaa att cag tcc				643
Phe Arg Ala Met Leu His Phe Cys Glu Asn Pro Gly Lys Ile Gln Ser				
	170	175	180	
cgt gct gaa ctg ctg aag aaa atg acc ggc cgt gag ctg aaa ccg cac				691
Arg Ala Glu Leu Lys Lys Met Thr Gly Arg Glu Leu Lys Pro His				
	185	190	195	
gac cgt act gta gac gtg acg atc cgc cgt att cgt aaa cat ttc gaa				739
Asp Arg Thr Val Asp Val Thr Ile Arg Arg Ile Arg Lys His Phe Glu				
	200	205	210	
tct acg ccg gat acg ccg gaa atc atc gcc acc att cac ggt gaa ggt				787
Ser Thr Pro Asp Thr Pro Glu Ile Ile Ala Thr Ile His Gly Glu Gly				
	215	220	225	
tat cgc ttc tgc ggt gat ctg gaa gat taa tcggctttac caccgtcaaa				837
Tyr Arg Phe Cys Gly Asp Leu Glu Asp				
	230	235		
aaaaacggcg ctttttagcg ccgtttttat ttttcaacct tatttccaga tacgtaaactc				897
atcgctcgtt gtaacttctt tactggcttt				927

[0099]

<210> 18

<211> 238

<212> PRT

<213> Escherichia coli

<400> 18

Met Gln Thr Pro His Ile Leu Ile Val Glu Asp Glu Leu Val Thr Arg

40

1	5	10	15
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20	25	30	
Ala Thr Asp Gly Ala Glu Met His Gln Ile Leu Ser Glu Tyr Asp Ile			
35	40	45	
Asn Leu Val Ile Met Asp Ile Asn Leu Pro Gly Lys Asn Gly Leu Leu			
50	55	60	
Leu Ala Arg Glu Leu Arg Glu Gln Ala Asn Val Ala Leu Met Phe Leu			
65	70	75	80
Thr Gly Arg Asp Asn Glu Val Asp Lys Ile Leu Gly Leu Glu Ile Gly			
85	90	95	
Ala Asp Asp Tyr Ile Thr Lys Pro Phe Asn Pro Arg Glu Leu Thr Ile			
100	105	110	
Arg Ala Arg Asn Leu Leu Ser Arg Thr Met Asn Leu Gly Thr Val Ser			
115	120	125	
Glu Glu Arg Arg Ser Val Glu Ser Tyr Lys Phe Asn Gly Trp Glu Leu			
130	135	140	
Asp Ile Asn Ser Arg Ser Leu Ile Gly Pro Asp Gly Glu Gln Tyr Lys			
145	150	155	160
Leu Pro Arg Ser Glu Phe Arg Ala Met Leu His Phe Cys Glu Asn Pro			
165	170	175	
Gly Lys Ile Gln Ser Arg Ala Glu Leu Leu Lys Lys Met Thr Gly Arg			
180	185	190	
Glu Leu Lys Pro His Asp Arg Thr Val Asp Val Thr Ile Arg Arg Ile			
195	200	205	
Arg Lys His Phe Glu Ser Thr Pro Asp Thr Pro Glu Ile Ile Ala Thr			
210	215	220	
Ile His Gly Glu Gly Tyr Arg Phe Cys Gly Asp Leu Glu Asp			
225	230	235	

[Brief Explanation of the Drawings]

[Fig. 1] Fig. 1 shows accumulation patterns in WC196, WC196 $\Delta$ arcA, WC196 $\Delta$ adam and WC196 $\Delta$ fnr.



[Name of Document] ABSTRACT

[Abstract]

[Object] To improve productivity of target substances including L-amino acids in fermentation production using  $\gamma$ -proteobacterium.

[Solving Means] In a method for producing a target substance utilizing a microorganism, which comprises culturing a  $\gamma$ -proteobacterium in a medium to produce and accumulate the target substance in the medium or cells and collecting the target substance, there is used a strain in which the ArcA protein does not normally function in the cell by means of, for example, disruption of the *arcA* gene on the chromosome.

[Drawing Selected] Fig. 1

[Name of Document] DRAWINGS

[Fig. 1]

